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HTLV-1 Cell Lines Differ in Constitutively Activated Signaling Pathways That Can Be Altered by Cytokine Exposure

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Examination of signaling pathways used by HTLV-1-infected rabbit cell lines revealed differences between one, RH/K30, that mediates asymptomatic infection and another, RH/K34, that causes lethal experimental leukemia. Both lines are IL-2 independent; RH/K30 produces IL-4 while RH/K34 produces IL-10. Examination of the Jak/STAT (Janus kinase/signal transducer and activator of transcription) activation of the lines revealed constitutive phosphorylation of Jak1 in both. STAT6 phosphorylation, not previously reported for HTLV-1 cells, was observed in RH/K30; STAT1 and STAT3 were phosphorylated in RH/K34. Treatment with cytokines altered the activation of the STAT proteins: IL-2 induced STAT5 phosphorylation in both lines. Supernatant from RH/K34 or IL-10 induced STAT3 phosphorylation in RH/K30 cells. Supernatant from RH/K30 or IL-4 induced STAT6 phosphorylation in RH/K34 cells, which could be reversed with a Jak kinase inhibitor—AG-490.

Key Words: HTLV-1; Jak/STAT; cytokine; IL-4; IL-10.

INTRODUCTION

The human retrovirus HTLV-1 infects 10–20 million people worldwide. While 95% of infected people remain lifelong asymptomatic carriers of the virus, approximately 5% develop HTLV-1-associated disease (Bangham, 2000). HTLV-1 causes a highly fatal T-cell malignancy, adult T-cell leukemia/lymphoma (ATLL), and a variety of chronic inflammatory syndromes, of which HTLV-1-associated myelopathy/tropical spastic paraparasis (HAM/TSP) is the most notable (Franchini, 1995). The mechanisms of the variable pathogenicity induced by this retrovirus are not clear, though the availability of infectious molecular clones and practical animal models has allowed elucidation of the important functions of the viral proteins in the life cycle, in T-cell transformation, and in *in vivo* infectivity (Franchini, 1995).

Our studies have used two HTLV-1-transformed rabbit T-cell lines to investigate factors involved in viral pathogenicity: RH/K30 mediates asymptomatic infection and RH/K34 causes a fatal leukemia-like disease within 10 days after injection of the rabbits (Simpson *et al.,* 1996). Comparison of proviral nucleotide sequences from the two cell lines revealed greater than 99% identity, which suggests that the structure of the integrated virus is not the sole factor in the ability of RH/K34 to cause ATLL-like disease (Zhao *et al.,* 1993). Studies with infectious molecular clones and chimeric clones derived from these cell lines have identified genetic regions important to *in vivo* or *in vitro* virus production (Zhao *et al.,* 1995, 1996). The relationship between infectivity of virus constructs and the pathogenesis produced by the infected cells remains obscure. Initial assessments of cytokine production and cell protein phosphorylation revealed differences between the two cell lines. RH/K30 does not produce IL-10, while RH/K34 does; both cells express the receptor for IL-10 (IL-10R) (Kindt *et al.,* 1999). These findings suggest that differences in the activation state of molecules involved in critical signaling pathways may be important to the functional differences of the cell lines.

The presence of high levels of the IL-2 receptor alpha chain (IL-2Rα) is a common feature of ATLL cells and HTLV-1-transformed cell lines (Franchini, 1995). One of the well-documented signaling pathways mediated by IL-2R is Jak/STAT. Under physiological conditions, activation of the Jak/STAT proteins is triggered by cytokine binding to its cell surface receptor. This is followed by the phosphorylation of Jak proteins and the subsequent tyrosine phosphorylation of the cytokine receptor. The phosphorylated tyrosine residues in the receptor provide a docking site for STAT proteins. The bound STAT protein is phosphorylated by the receptor-associated Jak kinase. After phosphorylation, STAT proteins dimerize and translocate to the nucleus to activate transcription of downstream genes, leading to an altered cell phenotype (Darnell, 1997; Heim, 1999). Abnormal activation of the STAT proteins is a common characteristic of leukemias (Lin *et al.,* 2000). Constitutive activation of the IL-2R–Jak3/STAT5 signaling pathway is correlated with IL-2 independence of HTLV-1-transformed cell lines (Xu *et al.,* 1995; Migone

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et al., 1995; Mulloy *et al.,* 1998). Similarly, a study with uncultured *ex vivo* leukemic cells from HTLV-1 seropositive patients with ATLL also displayed constitutive activation of Jak3, STAT1, STAT3, and STAT5 (Takemoto *et al.,* 1997). These results suggest that activation of the IL-2R signaling pathway mediated by Jak/STAT may play a key role in transformation by HTLV-1.

The present study concerns the Jak/STAT signaling pathway and the corresponding cytokines in the two IL-2-independent cell lines, RH/K30 and RH/K34. We found constitutive phosphorylation of STAT1 and STAT3 in RH/K34 cells, whereas in RH/K30 only STAT6 displayed constitutive phosphorylation. In addition, IL-4 and IL-10, differentially expressed by RH/K30 and RH/K34, controlled protein phosphorylation. These results suggest that the Jak/STAT pathways are intact in both the lethal and the nonlethal HTLV-1 lines and that specific STAT proteins are activated in response to cytokines produced by the two cell lines.

RESULTS

Constitutive phosphorylation of Jak/STAT proteins in HTLV-1-transformed T-cell lines

The Jak/STAT activation status of two rabbit HTLV-1 cell lines (RH/K30 and RH/K34) was tested and compared to HTLV-1-infected (MT-2) and uninfected (A3.01) human T-cell lines. Cell lysates from the four cell lines were analyzed using antibodies specific for native or tyrosine-phosphorylated proteins of Jak1 and members of the STAT family (STAT1, STAT3, STAT5, and STAT6). As shown in Fig. 1A, Jak1 was tyrosine phosphorylated in RH/K30, RH/K34, and MT-2 but not in A 3.01 cells. STAT1 and STAT3 were phosphorylated in RH/K34 and MT-2 cells, but not in RH/K30 cells. Unexpectedly, STAT5, which is normally activated in IL-2-independent lines, was observed only in MT-2 cells, not in the rabbit cell lines. STAT6 was tyrosine phosphorylated only in the RH/K30 cell line. None of the Jak/STAT proteins tested in this experiment were phosphorylated in the HTLV-1-noninfected cell line, A3.01, although it expressed detectable levels of each native protein. The nature of the activation pathways was further explored.

Jak1 is associated with STAT1 and STAT3

Jak1, which was constitutively phosphorylated in both cell lines, activates STAT1 and STAT3. Because neither STAT1 nor STAT3 was activated in RH/K30, we examined the association of Jak1 with STAT1, STAT3, and STAT6. Jak1 was immunoprecipitated with anti-Jak1 antibody from RH/K30 and RH/K34 cell lysates; the precipitates were analyzed by immunoblotting with STAT antibodies. As shown in Fig. 1B, Jak1 coprecipitated with STAT1 and STAT3 in both RH/K30 and RH/K34 cells, despite the absence of activated STAT1 or STAT3 in RH/K30. We

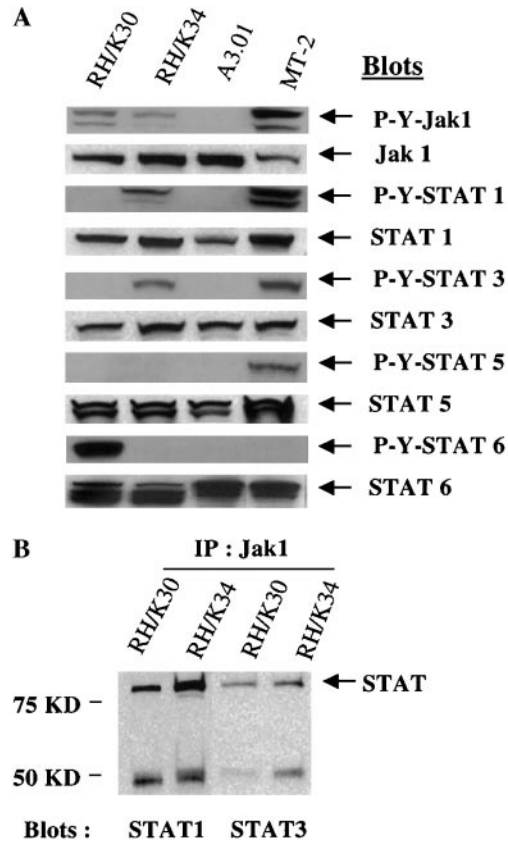

FIG. 1. (A) Tyrosine phosphorylation status of Jak/STAT proteins in HTLV-1-infected (RH/K30, RH/K34, and MT2) and noninfected (A 3.01) cell lines. Lysates from the cell lines were subjected to electrophoresis and immunoblotted with antibodies specific for native or tyrosine-phosphorylated forms of the Jak/STAT proteins as indicated to the right of each panel. (B) Jak1 is associated with STAT1 and STAT3. Jak1 was immunoprecipitated with anti-Jak1 antibody in RH/K30 and RH/K34 cells. Association with STAT proteins was determined by immunoblotting with STAT1 or STAT3 antibodies.

could not coprecipitate STAT6 with Jak1 in either cell line (data not shown), suggesting that other kinases were responsible for the phosphorylation of STAT6.

Expression of cytokines and their receptors by RH/K30 and RH/K34 cells

The Jak/STAT signal transduction processes are initiated by a variety of cytokines (Rane and Reddy, 2000) and HTLV-1 infection alters cytokine expression (Noma *et al.,* 1989; Villiger *et al.,* 1991; Mori *et al.,* 1996; Azimi *et al.,* 1998). Therefore, we determined the expression of cytokines and receptors in RH/K30 and RH/K34 cells by RT-PCR and flow cytometric analysis.

Binding experiments using biotinylated human IL-4 and IL-10 demonstrated that both cell lines express the receptors for IL-4 and IL-10 and can bind these cytokines at comparable levels (Fig. 2A). RT-PCR (Fig. 2B) confirmed that only RH/K34 expressed the gene for IL-10 and that IL-4 was expressed only in RH/K30. RT-PCR also

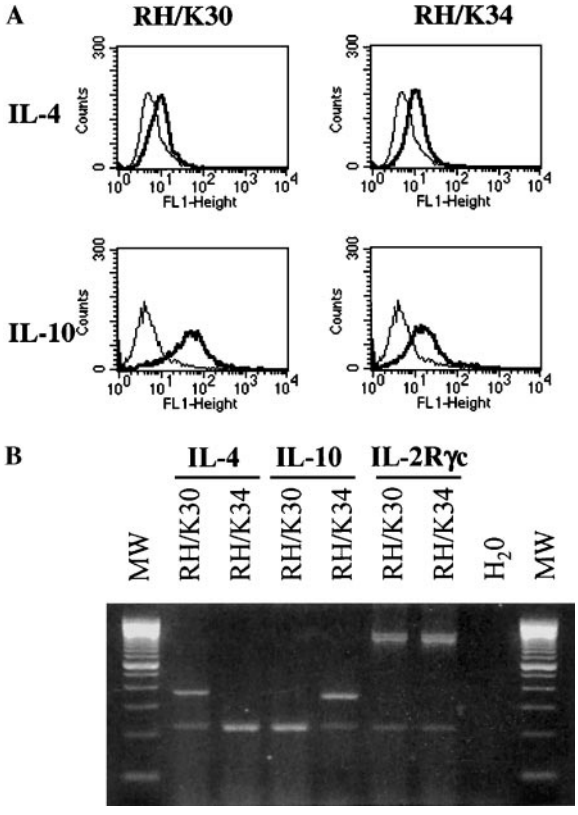

FIG. 2. Expression of IL-10 and IL-4 and cytokine receptors in HTLV-1-transformed cell lines. (A) FACS profiles of RH/K30 and RH/K34 showing binding of either biotinylated human IL-4 or biotinylated human IL-10 (depicted as boldface lines). Control fluorescence of the cells is depicted as light lines. (B) Agarose gel separation of RT-PCR products generated using primers for rabbit IL-4 (product size: 366 bp), IL-10 (product size: 348 bp), and human IL-2Rγc (product size: 1113 bp). Amplification of β-actin cDNA (product size: 218 bp) served as an internal control. The MW marker in this experiment is 100-bp DNA ladder.

indicated that both RH/K30 and RH/K34 expressed the IL-2Rγc gene (Fig. 2B). β-actin cDNA was PCR amplified in the RT reactions to serve as an internal control (Fig. 2B). These data suggested that the cytokine expression profile was different between the two lines although they had similar cytokine receptor expression patterns.

Cytokine-inducible phosphorylation of STAT3 in RH/K30 and STAT6 in RH/K34 cells

IL-10 is involved in the activation of STAT3, and IL-4 induces phosphorylation of STAT6 (Rane and Reddy, 2000). Because both lines express receptors for both cytokines but express only one of them, the inductive effect of IL-10 and IL-4 to stimulate the phosphorylation of STAT proteins was tested. As shown in Fig. 3A, human recombinant IL-10 induced the phosphorylation of STAT3 in RH/K30 cells but did not alter the phosphorylation level of STAT3 in RH/K34 cells. We also demonstrated that IL-4 induced phosphorylation of STAT6 in RH/K34 cells but at a lower level than achieved in similarly treated A3.01 cells. Blots using antibodies for either STAT3 or STAT6 showed equivalent loading of the gels and confirmed that the changes in tyrosine phosphorylation did not reflect an increase in protein expression levels (Figs. 3A and 3B, bottom). These results confirmed that the signaling pathways were intact in the two cell lines.

Results similar to IL-4/IL-10 induction were obtained using filtered supernatants from the two cell lines. As shown in Fig. 3C, supernatant from RH/K34 cells induced phosphorylation of STAT3 in RH/K30 cells. Conversely, supernatant from RH/K30 cells induced phosphorylation of STAT6 in RH/K34 cells within 1 h and the induction was dose-dependent (Fig. 3D). These results indicate that the specific phosphorylation of STAT proteins is induced by different cytokines.

The time course of the stimulation of tyrosine phosphorylation of STAT3 and STAT6 was examined next. RH/K30 cells or RH/K34 cells were stimulated for 5, 15, 30, 60, and 120 min with IL-10 or IL-4. As shown in Fig.

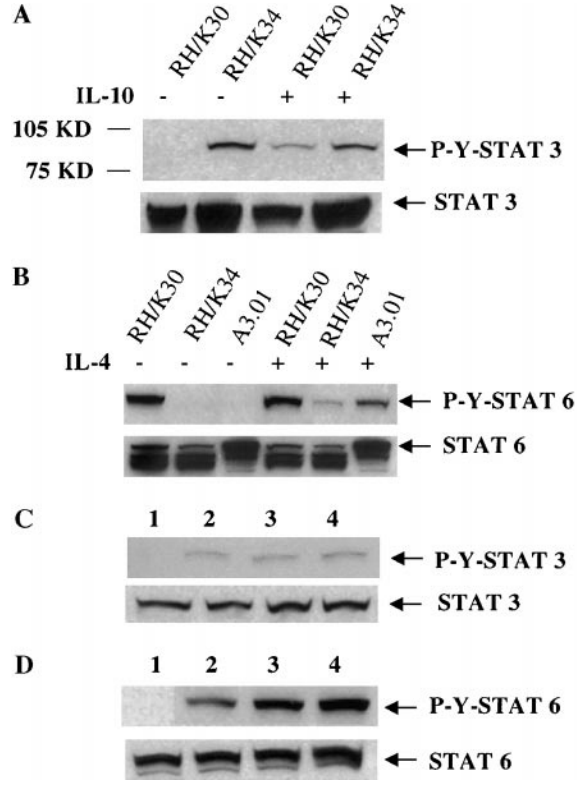

FIG. 3. Cytokine-induced tyrosine phosphorylation of STAT family proteins in HTLV-1-transformed cell lines. (A) Detection of STAT3 and tyrosine-phosphorylated STAT3 in cells lines untreated or treated with IL-10 (100 ng/ml) by immunoblotting. (B) Detection of STAT6 and tyrosine-phosphorylated STAT6 in cells lines untreated or treated with IL-4 (25 ng/ml) by immunoblotting. (C) RH/K30 cells were treated with RPMI medium (lane 1) and 20% (lane 2), 50% (lane 3), or 100% (lane 4) supernatant from RH/K34 cells for 1 h and native or phosphorylated STAT3 was tested by immunoblotting. (D) RH/K34 cells were treated with RPMI medium (lane 1), and 20% (lane 2), 50% (lane 3), or 100% (lane 4) supernatant from RH/K30 cells for 1 h and native or phosphorylated STAT6 was tested by immunoblotting.

4A, increased tyrosine phosphorylation of STAT3 in RH/K30 was detected as early as 5 min after stimulation and increased in a time-dependent manner until at least 120 min. The phosphorylation of STAT6 in RH/K34 was also induced as early as 5 min after IL-4 stimulation and reached its peak at 120 min (Fig. 4B). The phosphorylation of STAT6 persisted for as long as 24 h after stimulation (data not shown). Neither IL-4- nor IL-10-induced STAT phosphorylation reached the constitutive levels observed in RH/K30 or RH/K34 cells.

Effect of IL-2 on phosphorylation of STAT proteins

Because IL-2 plays a key role in HTLV-1 transformation, the effects of this cytokine were tested. Both RH/K30 and RH/K34 cells are IL-2-independent and express neither IL-2 nor phosphorylated STAT5. Addition of exogenous IL-2 induced phosphorylation of STAT5 in both lines with higher levels in RH/K34 cells (Fig. 5A). IL-2 did not induce the phosphorylation of either STAT1 in RH/K30 cells or STAT6 in RH/K34 cells; however, it did increase the levels of phosphorylation of STAT1 and STAT6 (Figs. 5B and 5C). Surprisingly, IL-2 neither induced the phosphorylation of STAT3 in RH/K30 cells nor increased the level of STAT3 phosphorylation in RH/K34 cells (Fig. 5D). Addition of IL-15 induced STAT5 phosphorylation in a manner similar to IL-2 (data not shown).

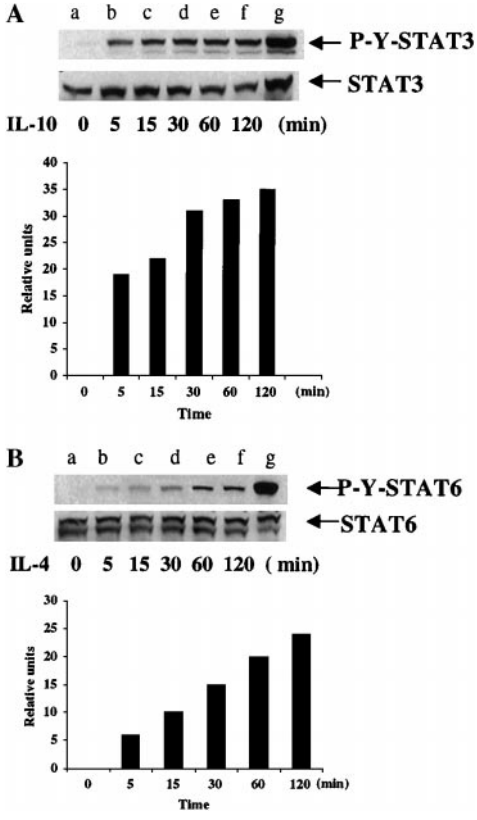

FIG. 4. Time course of STAT protein phosphorylation in rabbit HTLV-1 cell lines. (A) Detection of STAT3 or phosphorylated STAT3 in RH/K30 cells treated with IL-10 (100 ng/ml) for up to 2 h (lanes a–f) compared with untreated RH/K34 cells (lane g) by immunoblotting. Densitometric analysis of induced phosphorylation of STAT3. Intensity is plotted as relative units versus time of incubation. (B) Time course of IL-4- (25 ng/ml) inducible STAT6 phosphorylation in RH/K34 cells (lanes a–f) compared with STAT6 phosphorylation in untreated RH/K30 cells (lane g). Densitometric analysis of induced phosphorylation of STAT6. Intensity is plotted as relative units versus time of incubation.

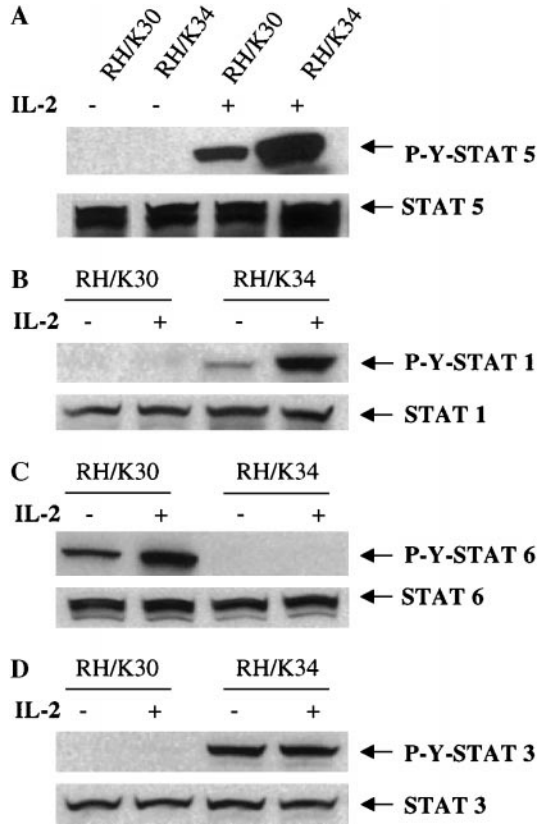

FIG. 5. Effects of IL-2 treatment on phosphorylation of STAT proteins in RH/K30 and RH/K34 cells. Cells were untreated or treated for 15 min with 2 nM IL-2 and tested for the presence of tyrosine phosphorylated (top) or native (bottom) STAT5 (A), STAT1 (B), STAT6 (C), or STAT3 (D) by immunoblotting.

Inhibition of STAT protein phosphorylation by AG-490

Tyrphostin AG-490, a derivative of benzylidine malononitrile, selectively blocks Jak2 and Jak3 kinases, but fails to inhibit Jak1, Tyk2, or other tyrosine kinases expressed in lymphocytes, including Lck, Lyn, Btk, Syk, Src, and Zap 70 (Meydan *et al.,* 1996; Kirken *et al.,* 2000). To assess AG-490 inhibitory effects on the constitutive or induced phosphorylation of Jak/STAT proteins, RH/K30 and RH/K34 cells were treated with AG-490 for 16 h and stimulated with the indicated cytokines. As shown in Fig. 6A, at 75 μM AG-490 markedly inhibited phosphorylation of STAT6 in RH/K30 cells, but only slightly inhibited phosphorylation of STAT1 in RH/K34 cells (Fig. 6B). It had almost no inhibitory effect on the phosphorylation of STAT3 in RH/K34 (Fig. 6C). As expected, AG-490 could

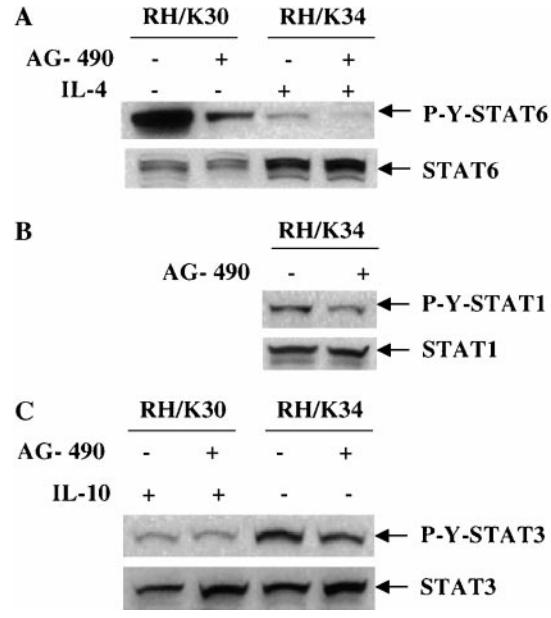

FIG. 6. Specific inhibition of constitutive or induced STAT phosphorylation by treatment with 75 μM AG-490. (A) RH/K30 or RH/K34 cells pretreated for 16 h with 75 μM AG-490 or with DMSO (control) were tested for tyrosine-phosphorylated STAT6 in the presence or absence of IL-4 (25 ng/ml). (B) RH/K34 cells were treated with 75 μM AG-490 or DMSO solvent control for 16 h and tested for tyrosine-phosphorylated STAT1. (C) RH/K30 or RH/K34 cells pretreated for 16 h with 75 μM AG-490 or with DMSO (control) were tested for tyrosine-phosphorylated STAT3 in the presence or absence of IL-10 (100 ng/ml).

inhibit IL-4-induced phosphorylation of STAT6 in RH/K34 cells (Fig. 6A), while it had no effect on IL-10-induced phosphorylation of STAT3 in RH/K30 cells (Fig. 6C). These data indicate that the HTLV-1 lines studied here utilize the conventional pathways of signal transduction despite the unusual observation of activated STAT6 in the RH/K30 cell line and the absence of the constitutive activation of STAT5, which is expected for IL-2-independent cell lines.

DISCUSSION

Abnormal STAT activation is a common feature of many solid and hematologic malignancies, reflecting the critical role of STAT proteins in regulating cell growth and differentiation. Constitutive STAT activation has been reported for HTLV-1-transformed cell lines and HTLV-1-infected ATLL patients (Migone *et al.,* 1995; Kirken *et al.,* 2000; Collins *et al.,* 1999; Zhang *et al.,* 1999; Takemoto *et al.,* 1997). The present study provides evidence for differences in phosphorylation of STAT proteins in HTLV-1-transformed rabbit cell lines with demonstrated differences in their ability to cause leukemia-like disease. The cell line RH/K30, which causes infection but no overt disease, displayed constitutive phosphorylation of STAT6; this has not been reported for other HTLV-1 cell lines or for primary ATLL cells. The more conventional pattern of STAT1 and STAT3 phosphorylation was found for the cell line RH/K34, which can cause lethal leukemia in rabbits. In either cell line, the alternative Jak/STAT signaling pathway was readily activated by treatment with supernatants from the other cell line or by treatment with either IL-4 or IL-10. Surprisingly, STAT5, which is normally activated in HTLV-1 cells, is not phosphorylated in the two cell lines unless they are treated with exogenous IL-2. Both lines express IL-2Rα and IL-2Rγc.

A series of different cytokines is known to activate one or more different STAT proteins. STAT6 is activated only by IL-4 and IL-13, which share a receptor chain (Hou *et al.,* 1994). In contrast, STAT1, 3, 5A, and 5B are activated by many different ligands (Darnell, 1997; Rane and Reddy, 2000; Akira, 2000). Expression of numerous cytokines including IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-15, TNF-α, and GM-CSF has been reported in HTLV-1-infected T-cells (Noma *et al.,* 1989; Villiger *et al.,* 1991; Mori *et al.,* 1996; Azimi *et al.,* 1998). Of special relevance to the present study are reports that serum IL-10 was detected in 80% of the patients with acute ATLL, whereas it was undetectable in most patients with chronic ATLL and in asymptomatic carriers of HTLV-1 as well as in all healthy blood donors tested (Mori *et al.,* 1996; Mori and Prager, 1998). The IL-10 concentration correlated with the evolution of ATLL from the active phase to remission or from the chronic to acute phase. Since IL-10 may be inhibitory to the generation of antitumor immunity (Wang *et al.,* 1994; Beissert *et al.,* 1995) the above reports suggest that HTLV-1 cells producing IL-10 can escape host defenses and mediate lethal disease. Our finding that IL-10 expression is upregulated in RH/K34 cells but not in RH/K30 cells is in complete conformity with this possibility. Mechanisms of IL-10 upregulation and whether it alone leads to leukemogenesis in HTLV-1 infection require further investigation.

If IL-10 is a hallmark of a lethal HTLV-1 cell, a question arises concerning the role of IL-4 expression as seen in RH/K30. Reports of IL-4 expression in HTLV-1-infected cells have varied significantly. Noma *et al.* (1989) found IL-4 expression in some ATLL cells transformed by HTLV-1, while Mori *et al.* (1994) found no evidence of IL-4 mRNA expression in the freshly isolated leukemic cells of 11 ATLL patients by RT-PCR. Shimamoto *et al.* (1996) also showed that the peripheral blood mononuclear cells (PBMCs) from HTLV-1 carriers consistently produced large amounts of certain cytokines (IFN-γ, IL-1α, TNF-α) but not IL-4. Recent reports of sequences for rabbit cytokines (Perkins *et al.,* 2000) facilitated detection of the IL-4 message in RH/K30 by RT-PCR. The present results provide an opportunity to investigate the role of IL-4 in an experimental model.

Specific cytokine/cytokine receptor combinations lead to activation of the various STAT proteins. Therefore, the interaction of a specific cytokine with its receptor in HTLV-1 cell lines might maintain the phosphorylation of

STAT proteins. The following two lines of evidence support this possibility. First, the differing profiles of cytokine expression correspond to the different patterns of phosphorylation of STAT proteins in RH/K30 and RH/K34 cell lines. Second, results from FACS and RT-PCR reveal that both RH/K30 and RH/K34 express IL-2Rγc, IL-4R, and IL-10R. Here we provide evidence that phosphorylation of STAT3 in RH/K30 cells occurs in response to exogenous IL-10 and that IL-4 induces the phosphorylation of STAT6. IL-2 increased the phosphorylation level of STAT1 in RH/K34 cells and of STAT6 in RH/K30 cells but it induced neither the phosphorylation of STAT3 in RH/K30 cells nor that of STAT6 in RH/K34. These data differ from studies showing that STAT3 phosphorylation is associated with IL-2/IL-2R in HTLV-1 cells (Migone *et al.,* 1995; Collins *et al.,* 1999; Zhang *et al.,* 1999) and suggest that, in addition to IL-2, other cytokines control HTLV-1-mediated STAT activation. Since the IL-15 receptor has component chains in common with the IL-2 receptor, it is a likely candidate to substitute for IL-2. Preliminary studies indicate that both RH/K30 and RH/K34 express IL-15 mRNA and that exogenous IL-15 had a similar effect on the phosphorylation of STAT proteins as IL-2 (Liang and Hague, unpublished data).

In the time course experiment, either IL-4 or IL-10 stimulated phosphorylation of the corresponding STAT in as little as 5 min, suggesting that the STAT3 and STAT6 signaling pathways are intact in both cell lines. Upon appropriate cytokine treatment alternative pathways can be activated quickly in each cell line. These results increase the possibility that cytokine treatment might alter the phenotype of the distinct cell lines and further alter their function.

The phosphorylation of STAT can be catalyzed by Jak family kinases, intrinsic receptor tyrosine kinases, and other cellular tyrosine kinases such as c-src (Lin *et al.,* 2000). The well-documented kinases in HTLV-1 cells are Jak1 and Jak3 (Migone *et al.,* 1995; Takemoto *et al.,* 1997; Mulloy *et al.,* 1998; Collins *et al.,* 1999). In addition to constitutive phosphorylation of STAT1, STAT3, and STAT6, we also found phosphorylation of Jak1 in both cell lines by direct immunoblotting with antibody specific for tyrosine-phosphorylated Jak1. Immunoprecipitation experiments indicated that Jak1 associates with STAT1 and STAT3 in RH/K34 cells, suggesting that Jak1 is the upstream kinase of STAT1 and STAT3. Surprisingly, STAT proteins were also associated with Jak1 in RH/K30, although no phosphorylated STAT1 or 3 was detectable in this cell line. Possible explanations for this include the presence of negative regulators of STAT proteins such as SSI (STAT-induced STAT inhibitor) in RH/K30 cells that bind to phosphorylated Jak1 to block its kinase effect on STAT proteins (Losman *et al.,* 1999; Sporri *et al.,* 2001). Another possibility is that phosphorylation of Jak1 may not always cause phosphorylation of STAT1 and STAT3. In a recent report, Zhang *et al.* (1999) found that Jak3 was constitutively phosphorylated but STAT5 was not in two HTLV-1-transformed IL-2-independent cell lines. The Jak2/3 inhibitor AG-490 inhibited not only the constitutive phosphorylation of STAT6 in RH/K30 cells but also IL-4-induced phosphorylation of STAT6 in RH/K34 cells. This is difficult to explain because neither Jak2 nor Jak3 phosphorylation was seen in either of the rabbit cell lines. In a similar situation, Nielsen *et al.* (1997) reported that AG-490 could inhibit the constitutive phosphorylation of a slowly migrating form of STAT3 in mycosis fungoides cells and STAT3 was found to be associated with Jak3, although Jak3 was not constitutively activated. They suggest that Jak3 might be an important "docking" protein rather than the initial activator and that malignant transformation of mycosis fungoides cells involves either a novel AG-490-sensitive kinase or an oncogenic form of a known Jak protein. This result prompts further study of the Jak3 proteins in rabbit HTLV-1 cells.

In conclusion, we have shown that the HTLV-1-transformed cell lines with variable pathogenicity *in vivo* could activate different Jak/STAT signaling pathways. Whether the activation of STAT3- and STAT6-induced genes explains the variable pathogenic phenotypes in HTLV-1 infection is open to question. The fact that activation status may be altered with cytokines will allow further investigation of the relationship between cell activation status and pathogenicity using the RH/K30 and RH/K34 cell lines in a well-established rabbit model for HTLV-1 infection and disease.

MATERIALS AND METHODS

Cell lines

The RH/K30 and RH/K34 cell lines were derived by infection of rabbit PBMCs using the human HTLV-I-infected cell line, MT-2. (Sawasdikosol *et al.,* 1993). A3.01 is a human T-cell leukemia cell line. All cell lines were maintained in RPMI 1640 complete medium containing 10% fetal calf sera (FCS) (Invitrogen, Rockville, MD), 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 mg/ml).

Determination of cytokine and receptor expression

The expression of receptors for IL-10 and IL-4 was determined using biotinylated rhIL-4 or biotinylated rhIL-10 in the respective Fluorokine Cytokine Receptor Detection Kit (R&D Systems, Minneapolis, MN). In order to determine the expression of IL-4, IL-10, and IL-2Rγc genes in the cell lines RH/K30 and RH/K34, RT-PCR was performed. Total RNA was isolated using the Trizol reagent (Invitrogen, Rockville, MD). First-strand cDNA was oligo(dT) primed and synthesized using 100 units M-MLV

reverse transcriptase (Invitrogen) in a reaction mix containing 50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, and 10 mM DTT. The PCR mixture contained a total volume of 10 μl: 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin; 3.5% glycerol; 0.2 mM dNTP; a 0.5 μM concentration of each forward and reverse primer; 1 μl (0.33 U) of *Taq* DNA polymerase (Perkin–Elmer, Norwalk, CT); and 1 μl (0.2–0.5 μg) of cDNA. Primer sequences were as follows: IL-10 F: 5′ GTG AAG ACT TTC TTT CAA ATC AAG G3′; IL-10 R: 5′GTT GAT GAA GAT GTC AAA CTC3′; IL-4 F: 5′ACA TCA TCC TAC CCG AAG3′; IL-4 R: 5′AAT ATT CAG CTC TGA CGC3′; IL-2Rγc F: 5′GCC ATG TTG AAG CCA TCA TTA CCA3′; IL-2Rγc R: 5′TCA GGT TTC AGG CTT TAG GGT3′. The primers β-actin F (5′TAC ATG GCT GGG GTG TTG AA3′) and β-actin R (5′AAG AGA GGC ATC CTC ACC CT3′) were included in all PCRs to amplify an internal positive control PCR product, a 218-bp fragment of β-actin. After denaturation for 5 min at 95°C, cDNA samples were subjected to 35 cycles of PCR in a DNA Thermal Cycler. Each cycle included 95°C for 30 s, 57°C for 30 s, and 72°C for 1.5 min followed by a final extension at 72°C for 5 min. PCR products were analyzed by electrophoresis on a 2% agarose gel containing 0.5 μg/ml ethidium bromide and then visualized using UV transillumination.

Cell treatment and cell lysate preparation

For the AG-490 inhibition experiment, cells were pretreated with varying concentrations of AG-490 (Calbiochem, La Jolla, CA) or DMSO as control for 16 h in RPMI 1640 medium with 1% FCS before exposure to cytokines. Cells were then stimulated with different cytokines as described in the figure legends. To test cytokine inductive effect, cells were washed once in serum-free RPMI 1640 medium and incubated in serum-free RPMI 1640 medium for 20–24 h. The next day, the cells were stimulated with 2 nM hrIL-2 (Calbiochem) for 15 min at 37°C or 25 ng/ml of hrIL-4 (Calbiochem) or 100 ng/ml of hrIL-10 (R&D Systems Inc.) for different time points at 37°C in 4 ml serum-free RPMI medium. After stimulation, cells were washed two times in cold PBS and lysed in 0.2 ml of lysis buffer containing 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM Tris–HCl, pH 7.4, protease inhibitor cocktail tablet (Roche, Indianapolis, IN), and phosphatase inhibitor cocktail (Sigma Co., St. Louis, MO) at 4°C with end-over-end shaking for 30 min. Samples were then centrifuged for 10 min at 13,000 rpm at 4°C, supernatants were transferred to a clean tube, and pellets were discarded. Protein concentration was measured with the DC protein assay kit (Bio-Rad, Hercules, CA). Samples were diluted to 2 mg/ml with the above lysis buffer and stored at −80°C or directly used for immunoblotting.

Immunoprecipitation and immunoblotting

For immunoprecipitation, samples were precleared with protein A–agarose (KPL, Gaithersburg, MD) for 1 h at 4°C. Beads were pelleted by centrifugation at 13,000 rpm for 1 min and supernatants were transferred to a fresh tube at 4°C. Primary antibody agarose conjugates (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were added to supernatants and the mixture was incubated at 4°C overnight with end-over-end rotation. Next, samples were centrifuged at 13,000 rpm for 1 min at 4°C and supernatants were removed. The beads were then washed three times with 1 ml of the above lysis buffer without protease inhibitor. Following washing, 40 μl of 2× SDS sample buffer (Invitrogen) was added with 5% 2-ME and samples were heated for 4 min at 95°C. Supernatants were then collected by centrifugation and 20 μl was loaded to the precast gel.

For the direct immunoblotting, the samples were thawed on ice, mixed with 6× SDS sample buffer (0.35 M Tris–HCl (pH 6.8), 10% SDS, 36% glycerol, 0.025% bromophenol blue, 5% 2-ME), and heated at 95°C for 4 min; 20-μl aliquots were then loaded to a precast Tris glycine gel (Invitrogen) and separated by SDS–PAGE at 130 V for 1.5 h. Following electrophoresis, proteins were transferred to Immobilon P membranes (Millipore, Bedford, MA) at 100 mA for 1.5 h. The membranes were incubated in blocking buffer containing PBS, 0.05% Tween 20, and 4% nonfat milk and then probed with specific primary antibodies for 2 h at room temperature or overnight at 4°C. Antibodies to STAT1, STAT3, STAT5, and STAT6 were purchased from Transduction Laboratories (Lexington, KY). Anti-Jak1 or anti-phosphotyrosine Jak1 antibodies were from Biosource International (Camarillo, CA). Anti-phosphotyrosine STAT3 was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-phosphotyrosine STAT1, STAT5, and STAT6 were purchased from New England Biolabs Inc. (Beverly, MA). Membranes were rinsed in PBST buffer (0.05% Tween 20 in PBS) for 5× 5 min and incubated with horseradish peroxidase-conjugated secondary antibody (Southern Biotechnology Associates, Birmingham, AL). Membranes were rinsed again for 5× 5 min in PBST buffer and developed with the enhanced chemiluminescence (ECL) immunoblotting system (Amersham Pharmacia Biotech, Piscataway, NJ).

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